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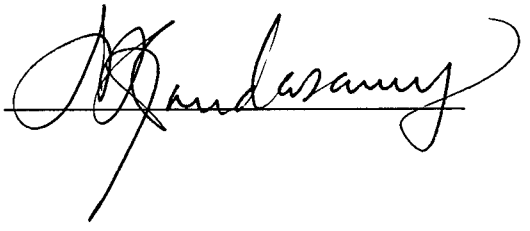

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13. ABSTRACT ***(Maximum 200 Words)***

The objective of this project was to determine whether hyaluronan interactions are involved in growth and invasion of tumor cells, especially mammary carcinoma. Our working hypothesis is that polymeric hyaluronan interacts in a multivalent manner with cell surface receptors such as CD44, thus inducing clustering of these receptors and concomitant intracellular signaling that leads to altered cell behavior typical of tumor cells. To test this hypothesis, two means of perturbing hyaluronan-protein interactions in vitro and in vivo have been employed in our studies. First, cDNA transfection and recombinant adenovirus infection have been used to over-express soluble hyaluronan-binding proteins in tumor cells; these proteins would be expected to act as a sink that competes for binding of endogenous hyaluronan. Second, tumor cells have been treated with hyaluronan oligomers; the oligomers compete for multivalent binding of endogenous polymeric hyaluronan by binding to receptors monovalently. Our results have shown that such perturbations of hyaluronan interactions inhibit tumor cell growth in vivo, anchorage-independent growth in soft agar, and invasion through extracellular matrix in vitro. The results of these studies provide strong evidence for the direct involvement of hyaluronan-tumor cell interactions in regulation of tumor growth and invasion.

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INTRODUCTION:

Many studies have implied that hyaluronan interactions at the surface of tumor cells may be a necessary part of the cascade of events leading to tumor progression. The objective of this project is to determine whether these hyaluronan interactions are involved in growth and invasion of tumor cells, especially mammary carcinoma, and if so to begin studies of the underlying mechanisms whereby hyaluronan has its effects. Our working hypothesis, based on previous work, is that polymeric hyaluronan interacts in a multivalent manner with cell surface receptors such as CD44 (1), thus inducing clustering of these receptors and concomitant intracellular signaling that leads to altered cell behavior typical of tumor cells (2,3). We reason that perturbation of endogenous hyaluronan binding reactions will lead to reversal of this altered cell behavior. Two means of perturbing hyaluronan-protein interactions in vitro and in vivo have been employed in our studies. First, cDNA transfection and recombinant adenovirus infection have been used to over-express soluble hyaluronan-binding proteins in tumor cells; these proteins would be expected to act as a sink that competes for binding of endogenous hyaluronan. Second, tumor cells have been treated with hyaluronan oligomers; the oligomers compete for multivalent binding of endogenous polymeric hyaluronan by binding monovalently to receptors (1); since polymeric hyaluronan is necessary for clustering of cell surface receptors, replacement with oligomers would reverse this clustering and consequent signaling events. Thus it is expected that both of these approaches would inhibit hyaluronan-induced changes associated with tumorigenesis.

From September 1, 1996, to June 30, 1999, Ms Rebecca Peterson examined the effects of over-expression of soluble CD44 on ascites growth in vivo and anchorage-independent growth in vitro, using murine mammary carcinoma cells. From October 1, 1999, to November 30, 2000, Ms Jeanine Ward studied the effects of over-expression of soluble hyaluronan-binding proteins and of treatment with hyaluronan oligomers on tumor cell invasion and anchorage-independent growth in culture.

BODY:

A. Work by Rebecca Peterson (09/01/96-06/30/99)

Task 1: Determine if transfection of soluble CD44 into murine mammary carcinoma cells (TA3/St cells) inhibits tumorigenesis in vivo.
Task 2: Determine if the inhibition of TA3/St tumorigenesis in vivo is dependent on hyaluronan binding to soluble CD44.
Task 3: Determine if there is a correlation between the sizes of hyaluronan that induce intracellular signaling in the tumor cells and that induce hyaluronan receptor clustering in their plasma membrane.

Tasks 1 and 2 have been completed. The data have been reported previously (1998 report) and published in Peterson et al (2000) Amer. J. Pathol. 156: 2159-2167 – see Appendix and summary of work in section A(i) below.

Task 3 was not feasible within the time frame of Ms Peterson's Ph.D. degree due to difficulties in producing well-characterized hyaluronan oligomer preparations of different sizes. This task was replaced by an investigation of the effect of over-expression of soluble CD44 on anchorage-independent growth in soft agar. This work has been completed, reported previously (1999 report), and published in Peterson et al (2000) Amer. J. Pathol. 156: 2159-2167 – see Appendix and summary of work in section A(ii) below.

A(i). Over-expression of soluble CD44 inhibits growth of TA3/St murine mammary carcinoma cells in ascites. Hyaluronan accumulates in ascites during intraperitoneal proliferation of TA3/St murine mammary carcinoma cells and at sites of their invasion of the peritoneal wall (4). To determine whether hyaluronan is functionally involved in these events, mice were injected intraperitoneally with stable transfectants of TA3/St cells that overexpress soluble CD44, a hyaluronan-binding protein that would be expected to compete with endogenous hyaluronan-protein interactions. The behavior of these transfectants was compared with that of transfectants expressing mutated soluble CD44 that does not bind hyaluronan. The soluble CD44 transfectants temporarily grew at a reduced rate within the peritoneal cavity, then went into G1 arrest and were subsequently cleared from the peritoneum. However, the transfectants overexpressing mutant soluble CD44 that does not bind hyaluronan exhibited similar ascites accumulation, growth rates and cell cycle profiles in vivo to that of wild type and vector-transfected TA3/St cells, both of which continued to grow until the tumors became fatal. The soluble CD44-transfected TA3/St cells also failed to attach to and form tumors in the peritoneal wall. These experiments indicate that perturbation of hyaluronan interactions by soluble CD44 alters the growth characteristics of the tumor cells, either directly or indirectly, and leads to inhibition of ascites growth and invasion in vivo. For further details, see Appendix: R.M. Peterson, Q. Yu, I. Stamenkovic, and B.P. Toole (2000) Amer. J. Pathol. 156, 2159-2167.

A(ii). Soluble CD44-transfected TA3/St mammary carcinoma cells have lost the capacity for anchorage-independent growth in vitro. It was not clear from the in vivo results obtained in the above study whether overexpression of soluble CD44 has a direct effect on tumor cell growth or whether its effect was an indirect consequence of another event in vivo. Thus additional evidence was sought to discriminate between these two possible explanations. First, proliferation of the soluble CD44 transfectants and control cells was compared in monolayer culture in tissue culture wells. Each cell line grew at approximately the same rate over a five day period and all cell lines exhibited similar cell cycle profiles. This result was in contrast to the differences observed previously between the growth of soluble CD44 transfectants in ascites vs that of controls in ascites, where the former were found to go into G1 arrest. Anchorage independent growth of the various cell lines in soft agar was then examined. Dramatic differences in size and number of colonies formed between the soluble CD44 transfectants and control cells were observed. The wild type, vector-transfected cells and mutant soluble CD44-transfected cells formed many times more colonies than the soluble CD44 transfectants, and the colonies formed by the control cells were much larger than those few colonies formed by the soluble CD44 transfectants. These results demonstrate that the

soluble CD44 transfectants, but not the mutant soluble CD44 transfectant, have lost their ability to exhibit anchorage independent growth in soft agar, one of the most reliable indicators for the transformed state of cells (5). We conclude that endogenous hyaluronan produced by the tumor cells themselves directly serves an important function in anchorage independent growth, and that hyaluronan interactions at the cell surface are, at least under some circumstances, crucial to mammary cancer cell growth characteristics in vitro and in vivo. For further details, see Appendix: R.M. Peterson, Q. Yu, I. Stamenkovic, and B.P. Toole (2000) Amer. J. Pathol. 156, 2159-2167.

B. Work by Jeanine Ward (10/01/99-11/30/00)

Task 1: Determine whether human cancer cells synthesize variant forms of soluble CD44 de novo.
Task 2: Construct and test recombinant adenoviruses driving expression of soluble hyaluronan-binding proteins.
Task 3: Determine whether infection of tumor cells with recombinant adenoviruses expressing various types of soluble hyaluronan-binding proteins, in addition to soluble CD44, inhibit tumor progression in vivo.

Task 1 is almost complete and is reported in section B(i) below. Our work to date on Task 2 is almost complete and is reported in section B(ii) below. The **in vivo** work for Task 3 has not been possible to complete in the time frame of the award. However, considerable progress has been made demonstrating inhibition of tumor cell growth and invasion **in vitro** by two recombinant adenoviruses expressing two different soluble hyaluronan-binding proteins; this is reported in section B(iii) below. Additional work showing that hyaluronan oligomers also inhibit tumor cell growth and invasion is also reported in B(iii). These results provide a strong basis for future experiments **in vivo**.

B(i). Human cancer cells synthesize soluble CD44 mRNA de novo. CD44 is usually expressed as a cell surface glycoprotein that, in many cell types, is present as numerous isoforms due to alternative splicing. Membrane-bound isoforms of CD44 contain common cytoplasmic, transmembrane and N-terminal hyaluronan-binding domains, but variable exon products are spliced into a single site in the membrane-proximal portion of the extracellular domain. Soluble forms of CD44 are present in the circulation and their levels are elevated in the sera of many tumor patients (6,7). The latter observation, together with the finding that over-expression of soluble CD44 protein inhibits growth of several tumor types in vivo, has led to the suggestion that soluble CD44 may act as an antagonist of membrane CD44. Cell culture studies by other laboratories suggest that soluble CD44 can be generated, at least in part, by proteolytic cleavage of membrane-bound CD44 (7). Our lab has shown in previous work that soluble CD44 is produced in embryonic murine tissues by alternative splicing, thus providing a mechanism of production de novo that is subject to rigorous cellular control (8). These murine soluble CD44 isoforms arise by alternative splicing of a newly discovered exon that lies between the previously described variant exons v9 and v10; this additional exon

includes a stop codon (8). Some murine soluble CD44 isoforms contain an additional novel domain arising from a 3' extension of exon v9, termed v9b (8). Thus, truncated CD44 isoforms lacking transmembrane and cytoplasmic domains are synthesized then secreted rather than inserted into the plasma membrane.

We have now shown that various types of human tumor cells also produce soluble isoforms of CD44 by alternative splicing of novel exonic sequences between the previously described v9 and v10 exons. However, the new sequences are quite different from those in the mouse. The human sequences occur as a 3' extension of v9 and a 5' extension of v10. The extension of human v9 includes a region homologous to murine v9b and a new exonic region, v9c, the homologue of which is present within an intron in the mouse. The 5' extension of human v10 is also intronic in the mouse. Despite the differences in arrangement of the mouse and human CD44 genes described above, the new exonic sequences between v9 and v10 give rise to stop codons that would lead to secreted isoforms of CD44 in both species. Since the alternative splicing that gives rise to soluble CD44 isoforms occurs 3' of v9 in both species, many variant isoforms of soluble CD44 can be produced using exons v2-v9 in the human and v1-v9 in the mouse. We have characterized transcripts for several different variant isoforms of soluble CD44 both in the mouse embryo and in human tumor cells. We have shown translation of secreted, soluble forms of CD44 from such transcripts in mouse cells but not yet in human. The physiological role of these secreted CD44 isoforms is not yet established but it is possible that they may act as antagonists or modulators of membrane-bound CD44 function (7,9). Indeed, recent studies in our laboratory show that overexpression of soluble CD44 isoforms in murine tumor cells inhibits tumor growth (section A above, and Appendix) and metastasis (2) in vivo, highlighting the potential importance of soluble isoforms of CD44 in regulation of membrane-bound CD44 activity.

B(ii). Construction of recombinant adenoviruses driving expression of soluble hyaluronan-binding proteins. For efficient expression of hyaluronan-binding proteins in tumor cells, we decided to use recombinant adenovirus constructs. Several constructs have been made in the laboratory, two of which have been used successfully in this study. These are recombinant adenoviruses driving expression of soluble CD44 and soluble BEHAB, a soluble hyaluronan-binding fragment derived from the proteoglycan, brevican (10). The adenoviruses were produced by routine methods (11) in which cDNA for each protein was cloned into an appropriate vector, then co-transfected with adenovirus into 293 cells which allow replication of the attenuated virus. After homologous recombination, the plaques were harvested and the virus purified and amplified. After determining successful expression of protein on infection of 293 cells, the viruses were used to infect C6 rat glioma cells. In both cases the protein (soluble CD44 or BEHAB) was expressed at high levels and secreted into the medium after infection with the respective adenovirus. A recombinant adenovirus expressing β-galactosidase was used as a control.

B(iii). Perturbation of hyaluronan interactions inhibits anchorage-independent growth and invasiveness of tumor cells. We reported above that over-expression of soluble CD44 in TA3/St murine mammary carcinoma cells inhibits anchorage-independent growth in soft agar whereas over-

expression of mutated CD44 that does not bind hyaluronan has no effect. The most likely interpretation of these results is that soluble CD44 acts by displacing endogenous hyaluronan from cell surface binding proteins. To substantiate this conclusion we have now studied the effect of treatment with hyaluronan oligomers on growth in soft agar. Hyaluronan oligomers would be expected to compete for endogenous polymeric hyaluronan binding but, in contrast to polymeric hyaluronan, would not bind multivalently (1), a property that has been shown to be important in hyaluronan signaling (2,3).

First we examined the effect of oligomers in routine monolayer culture of TA3/St murine mammary carcinoma cells and of C6 rat glioma cells. Growth under these conditions was unaffected by the oligomers. However, in soft agar the oligomers (100 μg/ml) cause 50-70% inhibition of colony formation by these two cell types.

We have begun to examine the effect of: a. over-expression of soluble CD44 or BEHAB via infection with the respective adenoviruses (from B(ii) above), and b. treatment with hyaluronan oligomers, on tumor cell invasiveness, concentrating initially on glioma cells because of the importance of their invasive properties in malignancy. We have used three cell lines: rat C6, human A172 and human U87 glioma; these lines actively invade Matrigel, a widely used model for measuring invasiveness (12). Inclusion of hyaluronan oligomers (100$_\mu$g/ml) in the Matrigel causes 70-90% inhibition of invasion by the three cell types. Over-expression of soluble CD44 or BEHAB in C6 glioma cells causes 50-60% inhibition of invasion.

KEY RESEARCH ACCOMPLISHMENTS:

a. Demonstration that hyaluronan interactions are crucial to growth in vivo of murine mammary carcinoma cells in ascites
b. Demonstration that hyaluronan interactions are crucial to invasion in vivo of murine mammary carcinoma cells from ascites into the peritoneal wall
c. Demonstration that hyaluronan interactions are crucial to anchorage independent growth of murine mammary carcinoma cells and gliomal cells in vitro
d. Demonstration that hyaluronan interactions are crucial to gliomal cell invasiveness in vitro
e. Demonstration that transcripts for soluble variants of CD44 are synthesized de novo by human tumor cells

REPORTABLE OUTCOMES:

a. Training of two Ph.D. students in the field of cell biology of breast cancer.
b. Ph.D. in Cell, Molecular and Developmental Biology at Tufts University awarded to Rebecca Peterson, 1999.
c. Employment of Rebecca Peterson as an Instructor in Biology at Penn State University.
d. Contribution toward the Ph.D. degree for Jeanine Ward (expected December, 2001).
e. Publication: Peterson, R.M., Yu, Q., Stamenkovic, I., and Toole, B.P. (2000): Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine

mammary carcinoma cells in ascites. Amer. J. Pathol. 156: 2159-2167.

f. Presentations of posters by Rebecca Peterson at the annual meeting of the American Society of Cell Biology and the Era of Hope meeting, and by Jeanine Ward at the annual Miami winter symposium on Cancer.

g. Award of National Cancer Institute grant (R01 CA82867) to pursue work based in part on results described above

CONCLUSIONS:

The results of these studies provide strong evidence for the direct involvement of hyaluronan-tumor cell interactions in the regulation of tumor growth and invasion. Consequently, perturbation of the pathways whereby hyaluronan influences tumor cell behavior may provide new avenues for therapeutic intervention in human patients.

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APPENDIX:

Reprint of: Peterson, R.M., Yu, Q., Stamenkovic, I., and Toole, B.P. (2000): Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites. Amer. J. Pathol. 156: 2159-2167.

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Perturbation of Hyaluronan Interactions by Soluble CD44 Inhibits Growth of Murine Mammary Carcinoma Cells in Ascites

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Hyaluronan accumulates in ascites during intraperitoneal proliferation of TA3/St murine mammary carcinoma cells and at sites of their invasion of the peritoneal wall. To determine whether hyaluronan is functionally involved in these events, ascites tumor formation was compared in mice injected intraperitoneally with stable transfectants of TA3/St cells that overexpress soluble CD44, a hyaluronan-binding protein, *versus* in mice injected with transfectants expressing mutated soluble CD44 that does not bind hyaluronan. The soluble CD44 transfectants temporarily grew at a reduced rate within the peritoneal cavity, then went into G_1 arrest and were subsequently cleared from the peritoneum. However, transfectants overexpressing mutant soluble CD44 that does not bind hyaluronan exhibited similar ascites accumulation, growth rates, and cell-cycle profiles *in vivo* to wild-type and vector-transfected TA3/St cells, all of which continued to grow until the tumors became fatal. The soluble CD44-transfected TA3/St cells also failed to attach to and form tumors in the peritoneal wall. When grown *in vitro* in soft agar, the soluble CD44 transfectants exhibited a dramatic reduction in colony formation compared to wild-type, vector-transfected, and mutant soluble CD44-transfected TA3/St cells. Thus, perturbation of hyaluronan interactions by soluble CD44 has a direct effect on the growth characteristics of these tumor cells, leading to inhibition of anchorage-independent growth *in vitro* and ascites growth *in vivo*. *(Am J Pathol 2000, 156:2159–2167)*

Breast cancer cells metastasize directly through the vasculature to organs distant from the original tumor site, but they also invade and exfoliate into body cavities, especially the pleural space, where they grow in suspension within effusions.[1] The rapid accumulation of these effusions is believed to result from increased permeability of the vasculature lining such cavities under the influence of tumor cell products, eg, vascular endothelial growth factor.[2] The breast cancer cells eventually attach to and invade tissues lining the cavity wall. The tumor cells then gain access to the many blood vessels contained therein, leading to further dissemination of malignant cells to other organs.[3]

In a past study, we showed that hyaluronan accumulates in the ascites, and at initial sites of attachment and invasion of tumor cells at the mesothelial surface of the peritoneal wall, after introduction of murine ovarian tumor cells or mammary carcinoma cells into the peritoneal cavity of syngeneic mice.[4] Several types of malignant solid tumors contain elevated levels of hyaluronan, a ubiquitous glycosaminoglycan that contributes both to the structure of extracellular matrix and to cell-matrix interactions that influence cell behavior.[5,6] The enrichment of hyaluronan in tumors can result from increased production by tumor cells themselves[7,8] or from interactions between tumor cells and surrounding stromal cells that induce increased production by the latter.[9–11] High levels of hyaluronan correlate with tumor spread and with poor survival rates in human patients with a variety of tumor types,[12–15] and experimental evidence in animal models directly implicates hyaluronan in solid tumor progression.[16–20] In the present study our objective was to determine whether hyaluronan also contributes to ascites growth and tumor cell invasion of the peritoneal wall.

We have shown that stable transfection of TA3/St murine mammary carcinoma cells with cDNA encoding soluble CD44 prevents formation of metastatic nodules in the lung after introduction of the TA3/St cells into the vasculature.[17] In that study, soluble CD44 presumably acted as a competitive inhibitor of crucial hyaluronan-protein interactions because transfection with mutant soluble CD44 that does not bind hyaluronan had no effect

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on invasion and metastasis. This supposition was confirmed by experiments showing that soluble CD44 transfection prevents hyaluronan-mediated clustering of endogenous membrane CD44 that is in turn required for binding of gelatinase B (MMP-9) to the tumor cell surface and for invasiveness.[21] In the present study, we show that stable transfection of TA3/St cells with soluble CD44 not only inhibits tumor invasion but also prevents tumor cell proliferation in ascites and that this inhibition is because of a direct effect on growth characteristics of the tumor cells rather than, or in addition to, an indirect effect on other events *in vivo*. These changes in tumor cell growth characteristics depend on hyaluronan interactions because mutated soluble CD44 that does not bind hyaluronan does not cause these changes.

Materials and Methods

Cell Lines and Culture Conditions

The TA3/St cell line was established from an ascites subline originally derived from a spontaneous mouse mammary adenocarcinoma.[2,22] TA3/St cells were maintained by weekly passages in the peritoneal cavities of syngeneic, 4- to 6-week-old female A/Jax mice or in culture in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT). Transfected TA3/St cells were cultured in DMEM supplemented with 10% FBS and 0.5 mg/ml geneticin (G418 sulfate, Life Technologies, Inc., Grand Island, NY).

Transfection of TA3/St Cells with Soluble CD44 Constructs

Soluble CD44 constructs were prepared and analyzed as described previously.[17] For transfection, TA3/St cells were treated with either pCR3-Uni eukaryotic expression vector alone (InVitrogen Corp., San Diego, CA) or pCR3-Uni vector containing cDNAs encoding soluble CD44 isoforms, in the presence of lipofectamine. These isoforms included either variant exons v8-v10 or v6-v10, where v10 is a new insert containing a stop codon, thus leading to truncation before the transmembrane domain;[17,23] v6-v10 was used with or without the R43A mutation that leads to loss of hyaluronan binding capacity.[17,24] G418-resistant colonies were selected and seven clones were chosen for further study: two transfectants containing v6-v10 (v6-v10a and v6-v10b), one containing v8-v10, two containing the v6-v10 mutant (v6-v10 R43A), and two mock transfectants containing vector only. The transfectants were analyzed by reverse transcriptase-polymerase chain reaction, fluorescence-activated cell sorting, and Western blotting as described previously[17] to confirm that each clone expresses the appropriate CD44 protein. All transfectants and wild-type cells produced similar amounts of surface-associated, standard, and variant CD44 isoforms. However the soluble CD44 transfectants, including the mutant soluble CD44 transfectants, also produced soluble, secreted CD44.[17]

Tumorigenicity Assay

TA3/St cells in log phase growth were trypsinized, washed with DMEM containing 10% FBS, and resuspended in Hanks' balanced salt solution (HBSS; Life Technologies, Inc.) for counting. Suspensions of TA3/St cell lines were injected, using a 25-gauge needle, into the peritoneal cavities of 4- to 6-week-old female A/Jax mice (The Jackson Laboratory, Bar Harbor, ME) at 1×10^6 cells/200 μl HBSS each, and allowed to grow *in vivo* for a period of 7 to 19 days. For each cell line and time point, six mice were given injections. Mice were observed daily for signs of ascites tumor development and monitored twice daily after the tumor symptoms appeared: abdominal bloating, decreased movement, loss of grooming behavior, and hunched posture. If mice were not expected to survive overnight they were sacrificed before conclusion of the experimental protocol. Mice that did not exhibit the above symptoms were sacrificed according to experimental parameters. The peritoneal walls from each of the mice were removed, cut into strips (~4 mm × 8 mm), and fixed in 4% paraformaldehyde (Tousimis, Rockville, MD) in phosphate-buffered saline (PBS) for histological analysis.

Histology

Fixed strips of peritoneal wall were washed in PBS, dehydrated through 30%, 70%, 95%, 100% ethanol and xylene, and then embedded in paraffin wax (Fisher, Columbia, MD). Sections (5 μm) were cut, mounted on poly-L-lysine (Sigma)-coated slides, and stained with Mayers modified hematoxylin (Poly Scientific Research, Bay Shore, NY) or hematoxylin and eosin (Richard-Allen Medical, Richland, MI) after deparaffinization in xylene and rehydration through 100%, 95%, 70%, 35% ethanol, PBS, and water.

In Vivo *Cell Proliferation Assay*

Transfected TA3/St cells in log phase growth were trypsinized, washed with DMEM containing 10% FBS, and resuspended in HBSS for counting. Suspensions of the transfected TA3/St cells were seeded into the peritoneal cavities of female A/Jax mice at 2×10^6 cells/200 μl HBSS each and allowed to grow *in vivo* for a period of 2 to 15 days. At each of five different time points (2, 5, 7, 10, and 15 days), groups of six mice were sacrificed and cells were harvested from the peritoneal cavities with two 6-ml intraperitoneal lavages of calcium- and magnesium-free PBS (PBS^-; Life Technologies, Inc.). Harvested cells were then washed three times with PBS−, using low-speed centrifugation with each wash to remove any red blood cells that were withdrawn along with tumor cells from the peritoneal cavity; the tumor cells formed a pellet while the red blood cells remained in the supernatant during these centrifugations. The washed cells were counted in a Coulter Counter (Coulter Electronics, Hialeah, FL) by diluting aliquots of cells resuspended in PBS− to concentrations between 200 to 20,000 cells/ml.

Cell-Cycle Analyses

Transfected and wild-type TA3/St cells were grown intraperitoneally for 7 days, harvested, and washed as described in the previous section. They were then resuspended in 70% EtOH and kept at −20°C until all samples had been collected for cell-cycle analysis. After removal from the freezer, the cells were washed twice with PBS−, resuspended in PBS− containing 0.1 mmol/L EDTA, pH 7.4, 50 mg/ml propidium iodide, 50 mg/ml RNase A (Boehringer Mannheim, Indianapolis, IN), and 1% Triton X-100, and incubated overnight at 4°C. Cell samples were then analyzed by fluorescence-activated cell sorting in a FACScan (Becton Dickinson, Mountain View, CA).

Transfected and wild-type cells were cultured *in vitro* in DMEM plus 10% FBS, then harvested during log phase of growth, and analyzed in the same way as above.

In Vitro *Cell Proliferation Assay*

Each cell line, in log growth phase, was trypsinized, washed with DMEM containing 10% FBS, and resuspended in the same media for culture. Cells were plated at 5×10^4 cells per well in 6-well plates (60-mm wells) and allowed to grow in 4 ml of medium at 37°C for 1 to 5 days. Every 24 hours, triplicate wells for each cell line were trypsinized, washed with DMEM, and resuspended in PBS^-. The harvested cells were then counted in a Coulter Counter after dilution in PBS− to concentrations of 200 to 10,000 cells/ml.

Soft Agar Assay

Soft agar assays were performed in 60-mm dishes containing 2 ml of 1.2% agarose diluted with 2× DMEM containing 20% FBS to yield a final agarose concentration of 0.6%. Cells were harvested from monolayer culture in log growth phase by trypsinization, washing, and resuspension in DMEM containing 10% FBS for counting. The cells were then suspended in 0.33% agarose in DMEM containing 10% FBS and plated at 5000 cells/well on top of the 0.6% agarose base. After each agarose layer was allowed to solidify (10 minutes at 25oC), three additional 1-ml volumes of 0.33% agarose were layered on top of the cells. Each cell line was plated in triplicate and grown at 37°C for 28 days. Total numbers of colonies per well containing >30 cells or >200 cells per colony were counted separately using a microscope grid. The two classes of colony size were assessed by counting cells in numerous colonies under the microscope and correlating these numbers with colony size. The two classes could be distinguished readily because the great majority of colonies were found to contain between 30 and 100 cells; the large colonies (>200 cells) were very easily distinguished from the majority of colonies (30 to 100 cells) and there were virtually no colonies with <30 cells.

Results

Transfection of TA3/St Cells with Soluble CD44 cDNA

Stable transfectants overexpressing the naturally occurring soluble CD44 isoforms, v6-v10 and v8-v10,[23] the mutant isoform, v6-v10 R43A,[24] or vector alone were selected and analyzed for CD44 production and secretion as described in Materials and Methods. All cell lines produced similar amounts of membrane-bound CD44. Only the soluble CD44 transfectants, including the mutant soluble CD44 transfectants, produced secreted CD44; clones were selected that produced similar amounts of soluble CD44.[17] Binding of hyaluronan to the soluble CD44 transfectants and their adhesion to a hyaluronan substratum were shown previously to be reduced compared to wild-type TA3/St cells and vector controls.[17,21] Stable transfectants producing mutated soluble CD44 (v6-v10 R43A) exhibited high levels of hyaluronan binding and adhesion to hyaluronan, similar to wild-type and vector controls.[17,21]

Overexpression of Soluble CD44 Inhibits Growth in Ascites and Peritoneal Wall Invasion by TA3/St Mammary Carcinoma Cells

Syngeneic A/Jax mice were injected intraperitoneally, at 1×10^6 cells per animal, with wild-type TA3/St cells or with TA3/St transfectants expressing soluble CD44 isoforms, mutant soluble CD44 (v6-v10 R43A), or vector alone. Tumor growth and invasion were assessed as described in Methods. Because results obtained in pilot experiments were virtually identical for the wild-type cells, both vector transfectants and both mutant soluble CD44 transfectants, only one of each control transfectant was examined in detail. Three soluble CD44 transfectant clones, two expressing v6-v10 and one expressing v8-v10, were examined in detail. Table 1 summarizes the results of one such experiment in which ascites accumulation, tumor growth, and tumor invasion were compared in the above manner in groups of six animals that were injected with either wild-type TA3/St cells, one of the vector transfectants, one of the mutant soluble CD44 transfectants, or the two v6-v10 soluble CD44 transfectants. Identical results to those shown in Table 1 for the two v6-v10 transfectants were obtained for the v8-v10 soluble CD44 transfectant in other similar experiments. The results of these experiments are discussed below.

The mice injected with wild-type, vector-transfected, or mutant soluble CD44 (v6-v10 R43A)-transfected cells accumulated ascites fluid and exhibited abdominal bloating, symptomatic of ascites tumor development. Mice injected with either of the two v6-v10 soluble CD44 transfectants or with the v8-v10 transfectant did not accumulate ascites fluid or exhibit abdominal bloating. At 7 days or at 14 to 19 days after injection of cells, the mice were sacrificed and their peritoneal walls were fixed and stained. At seven days, tumor cells had attached to regions of the peritoneal wall in wild-type, vector-transfected, or mutant soluble CD44 (v6-

Table 1. Soluble CD44 Transfectants of TA3/St Mammary Carcinoma Cells Have Lost Their Tumorigenicity *in Vivo*

Cell type	Numbers of animals		
	Attachment*	Growth/invasion†	Ascites‡
Controls			
Wild-type TA3/St	6/6	6/6	6/6
Vector transfectant	6/6	6/6	6/6
Soluble CD44 v6–v10 R43A	6/6	6/6	6/6
Soluble CD44 transfectants§			
Soluble CD44 v6–v10a	0/6	1¶/6	0/6
Soluble CD44 v6–v10b	0/6	2¶/6	0/6

*Animals were examined for attachment to the peritoneal wall and mesentery at 7 days after injection (see Figure 1).
†Animals were examined for growth and invasion in the mesentery and peritoneal wall between 14 and 19 days after injection (see Figure 2).
‡Animals were examined for accumulation of ascites between 14 and 19 days after injection.
§The soluble CD44 v8–v10 transfectant was not analyzed in this particular experiment but in other similar experiments it behaved in an identical fashion to that shown here for the v6–v10 transfectants.
¶Limited, focal regions of tumor cell attachment resulting in small accumulations of noninvading cells.

v10 R43A)-transfected controls (Figure 1A). However, no tumor cells were observed attached to the peritoneal walls of mice injected with the v6-v10 or the v8-v10 soluble CD44 transfectants (Figure 1B). Mice injected with wild-type, vector-transfected, or mutant soluble CD44-transfected cells exhibited widespread tumorigenesis and invasion of the mesothelium and muscle layers of the peritoneal walls by 14 to 19 days (Figure 2; A, C, and D). In contrast, mice injected with v6-v10 or v8-v10 soluble CD44-transfected cells showed little or no tumor cell attachment to the mesothelium and no invasion of the peritoneal wall (Figure 2B).

Because transfection with soluble CD44 cDNA eliminated ascites accumulation and invasion of the peritoneal wall, we directly analyzed growth of the injected cells to determine their fate *in vivo*. To facilitate high cell recovery from the peritoneal cavity, a larger number of cells, ie, 2×10^6, was injected into the abdomen of each host mouse than in the experiments above. The cells were recovered by intraperitoneal saline lavage at 2, 5, 7, 10, and 15 days postinjection and counted (Figure 3). Vector-transfected and soluble CD44 R43A-transfected cells grew rapidly for the first 10 days after injection; however, most of these animals then became terminally ill and were sacrificed before day 15. Consequently only one to two animals remained for analysis at the 15-day point in these cases (in all other situations at least six animals were analyzed at each of the five time points). For the first 5 to 7 days, the soluble CD44 transfectants grew significantly, but at a diminished rate compared to the controls. Subsequently, between 10 and 15 days postinjection, their numbers became reduced to baseline (Figure 3). This rise and fall in number of cells is particularly evident for the transfectant overexpressing the v8-v10 soluble CD44 isoform, where the ascites cell number rose to $\sim 14 \times 10^6$ at day 7 after injection but fell back to baseline by 15 days (Figure 3). None of the animals carrying the soluble CD44 transfectants accumulated ascites and most survived indefinitely without any signs of tumor formation in the peritoneal wall. A small number of these animals slowly developed solid tumors outside the peritoneum near the site of tumor cell injection, presumably arising from cells that leaked from the peritoneum during injection or healing.

Soluble CD44-Transfected TA3/St Mammary Carcinoma Cells Enter G_1 Arrest in Ascites

Wild-type and transfected TA3/St cells were harvested at 7-days postinjection from the peritoneal cavity of mice

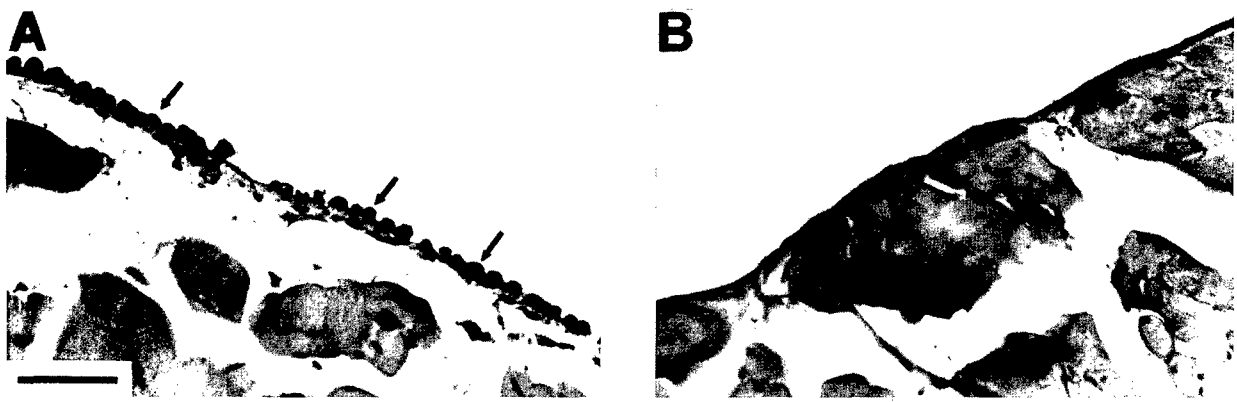

Figure 1. Soluble CD44 transfectants do not attach to the peritoneal wall *in vivo*. TA3/St transfectants were injected into the peritoneal cavity of syngeneic A/Jax mice (1×10^6 cells each), then the animals were sacrificed after 7 days, and their peritoneal walls were fixed and stained. **A**: Control TA3/St cells transfected with vector alone; **B**: TA3/St cells transfected with soluble CD44. The control cells attached to the peritoneal wall (**arrows**) whereas the soluble CD44 transfectants did not. Similar attachment to that shown in **A** for vector transfectants was also obtained with wild-type and mutant soluble CD44 (R43A)-transfected cells; no attachment was observed with soluble CD44 transfectants v6-v10a, v6-v10b (see Table 1), or v8-v10. Scale bar, 50 μm.

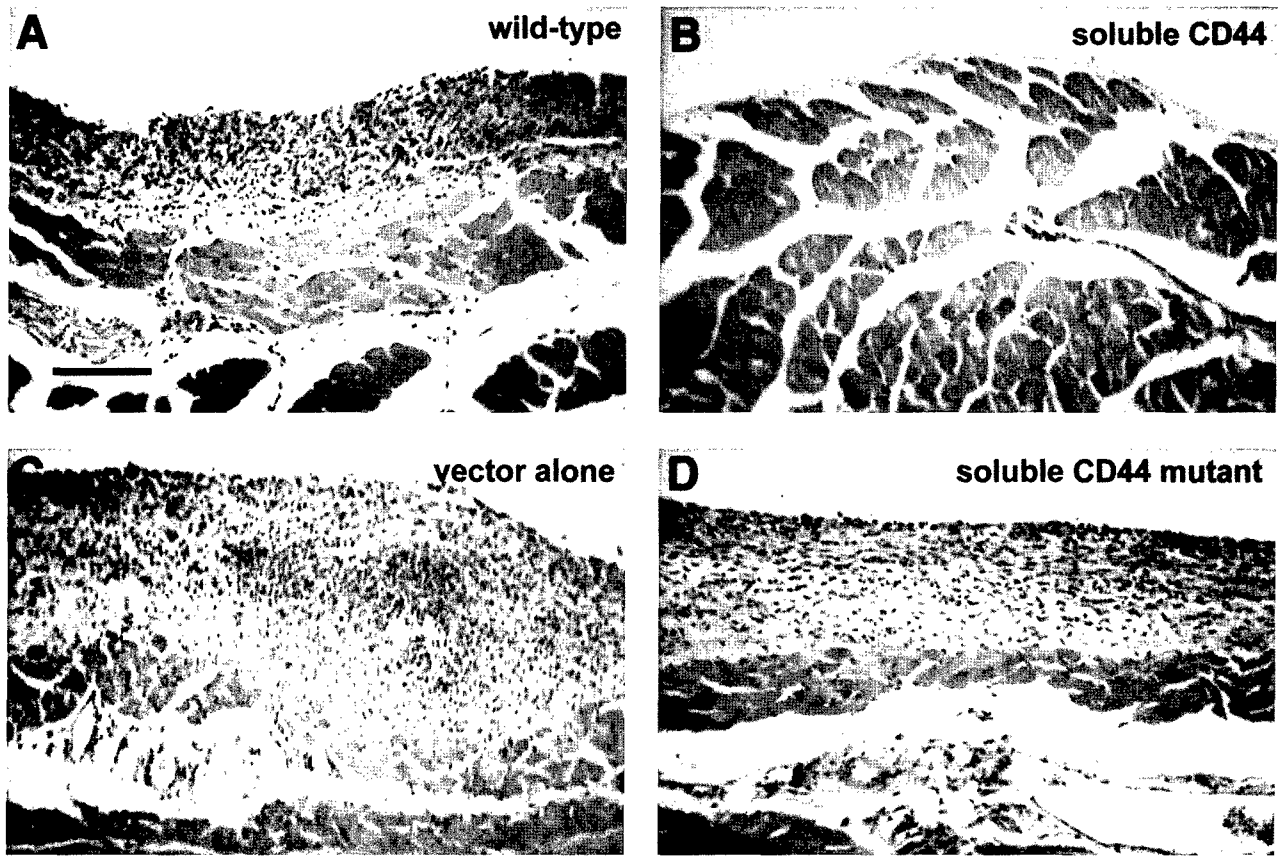

Figure 2. Soluble CD44 transfectants do not form tumors in the intraperitoneal wall. TA3/St transfectants expressing soluble CD44 or soluble CD44 with a point mutation (R43A) in the hyaluronan-binding domain (mutant soluble CD44), vector-transfectants, or wild-type TA3/St murine mammary carcinoma cells were injected into the peritoneal cavity of syngeneic A/Jax mice. At 14- to 17-days postinjection, the mice were sacrificed, and their peritoneal walls were fixed and stained. Mice injected with wild-type (**A**), vector-transfected (**C**), or mutant soluble CD44-transfected cells (**D**) exhibited widespread tumor growth and invasion of the mesothelium and muscle layers of their peritoneal walls. Mice injected with soluble CD44-transfected cells (**B**) were shown to have normal peritoneal walls without tumor cell growth and invasion or, in a few cases, a small number of tumor cells attached to the mesothelium; similar results were obtained with v6-v10a, v6-v10b (see Table 1), and v8-v10 transfectants. Scale bar, 100 μm.

injected with 2×10^6 cells. These cells were then analyzed by fluorescence-activated cell sorting to establish a cell-cycle profile for each cell line *in vivo*. Cells transfected with soluble CD44 isoforms exhibited G_1 arrest, whereas wild-type, vector-transfectant, and soluble CD44 R43A-transfected cells demonstrated a cell-cycle profile typical of an asynchronously cycling cell population (Figure 4; Table 2). The proportion of cells in G_0/G_1 for each population was calculated to be approximately 30 to 40% for the various control populations compared to 75 to 85% for the soluble CD44 transfectants (Table 2).

For comparison, the various cell lines were grown in monolayer tissue culture instead of in ascites. In this case, both the soluble CD44 transfectants and the control cell types exhibited similar cell-cycle profiles, ranging from ~30 to 50% cells in G_0/G_1 (Table 2).

In a further attempt to understand the fate of the soluble CD44-transfected TA3/St cells after inoculation into the peritoneum, we allowed the transfectants to grow *in vivo* for 5 days, checked that they had gone into G_1 arrest, as found above, then placed the cells in culture to see whether they would recover. The soluble CD44-transfected cells failed to attach and grow, whereas vector controls grew in similar fashion to that before inoculation *in vivo* (data not shown). Thus we conclude that, in as-

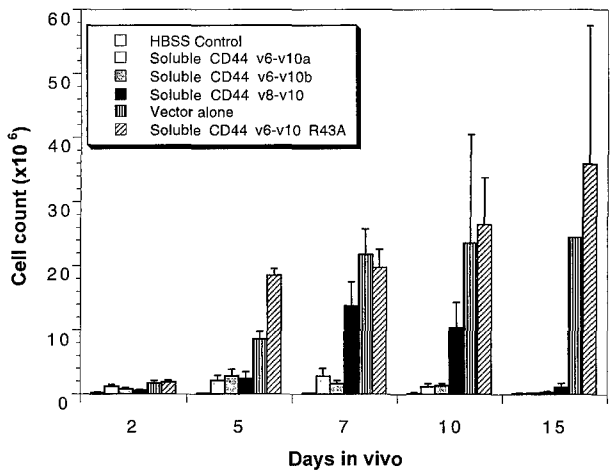

Figure 3. Overexpression of soluble CD44 alters tumor cell proliferation in ascites. Cell numbers were counted as described in Methods for soluble CD44-transfected, vector-transfected, and soluble CD44 R43A-transfected TA3/St cells throughout 15 days *in vivo*. The soluble CD44 transfectants (v6-v10a, v6-v10b, v8-v10) grew at a diminished rate and dropped back to baseline (HBSS control) between 10- to 15-days postinjection with 2×10^6 cells, whereas the vector-transfected and the soluble CD44 R43A-transfected cells continued to grow. Growth rates of cells within the peritoneal cavity of six mice per condition and time point are represented, except in the case of the vector-transfected and soluble CD44 R43A-transfected cells at 15-days postinjection, where many of the animals became ill and had to be sacrificed between days 10 and 15.

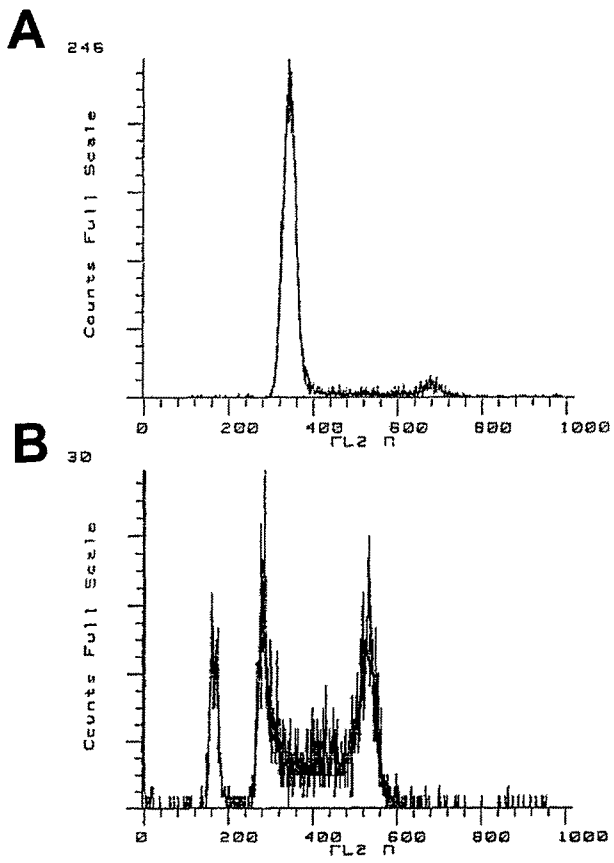

Figure 4. Soluble CD44 transfectants grown in ascites enter G_1 arrest. **A**: Cell-cycle analysis of soluble CD44-transfected cells grown in ascites; **B**: vector-transfected control. Quantitative data are given in Table 2. A similar cell-cycle pattern to that of the vector-transfected cells (**B**) was obtained with wild-type and mutant soluble CD44-transfected cells; the cell-cycle patterns seen with all three soluble CD44 transfectants, v6-v10a, v6-v10b, and v8-v10, were similar to that shown in **A** (see Table 2).

cites, the soluble CD44 transfectants irreversibly entered G_1 arrest, subsequently died, and were cleared from the peritoneum.

Soluble CD44-Transfected TA3/St Mammary Carcinoma Cells Have Lost the Capacity for Anchorage-Independent Growth in Vitro

It is not clear from the results obtained above whether overexpression of soluble CD44 has a direct effect on tumor cell growth or whether its effect was an indirect consequence of another event *in vivo*. Thus we sought additional evidence to discriminate between these two possible explanations.

First, proliferation of the soluble CD44 transfectants and control cells was compared in monolayer culture in tissue culture wells. Each cell line grew at approximately the same rate during a 5-day period (Figure 5) and exhibited similar cell-cycle profiles (Table 2).

We then examined anchorage-independent growth of the various cell lines in soft agar. Dramatic differences in size and number of colonies formed between the soluble CD44 transfectants and control cells were observed (Table 3). The wild-type, vector-transfected, and mutant soluble CD44-transfected cells formed many times more

Table 2. Soluble CD44 Transfectants Exhibit G_1 Arrest *in Vivo*

Cell type	% Cells in G_0/G_1	
	In vitro	*In vivo*
Controls		
Wild-type TA3/St	44.1 ± 0.5	30.0 ± 2.1
Vector transfectant	28.3 ± 1.5	38.2 ± 5.4
Soluble CD44 v6–v10 R43A	33.3 ± 0.9	39.1 ± 4.8
Soluble CD44 transfectants		
Soluble CD44 v6–v10a	44.7 ± 1.6	86.2 ± 1.0
Soluble CD44 v6–v10b	37.0 ± 2.1	76.4 ± 4.1
Soluble CD44 v8–v10	53.0 ± 1.2	74.3 ± 2.8

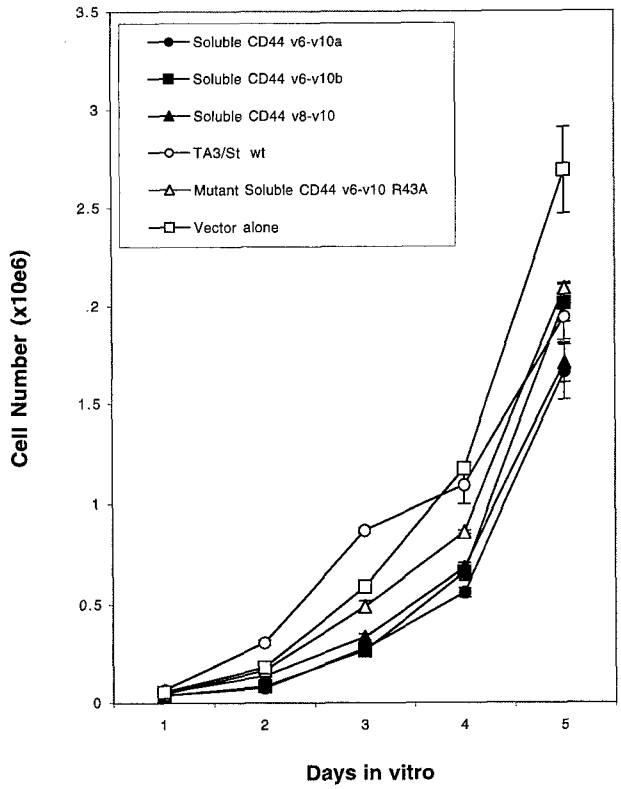

Figure 5. Overexpression of soluble CD44 does not affect tumor cell growth in monolayer culture. Cells were grown in standard monolayer conditions. Cell numbers were counted in triplicate cultures at each time point as described in Methods. No significant differences were observed among the various cell lines.

Table 3. Soluble CD44 Transfectants Fail to Form Colonies in Soft Agar

Cell type	Number of colonies*	
	>30 cells	>200 cells
Controls		
Wild-type TA3/St	205 ± 28	30 ± 2
Vector transfectant	83 ± 21	25 ± 9
Soluble CD44 v6–v10 R43A	409 ± 11	39 ± 5
Soluble CD44 transfectants		
Soluble CD44 v6–v10a	1 ± 0	0 ± 0
Soluble CD44 v6–v10b	16 ± 4	5 ± 1
Soluble CD44 v8–v10	8 ± 1	0 ± 0

*Numbers represent means (±SD) of the total numbers of colonies with >30 or >200 cells per colony in triplicate wells.

colonies than the soluble CD44 transfectants, and the colonies formed by the control cells were larger than those few colonies formed by the soluble CD44 transfectants (Table 3).

Discussion

In the work presented here we have demonstrated that stable transfection of malignant TA3/St mammary carcinoma cells with cDNAs encoding soluble CD44 isoforms directly alters their growth and adhesion characteristics such that they are unable to form ascites tumors or to invade the tissues of the host animal after intraperitoneal injection. Transfection with mutated cDNA encoding soluble CD44 that does not bind hyaluronan, and thus does not interfere with hyaluronan binding or cellular adhesion to hyaluronan,[17,21] failed to inhibit the tumorigenicity of TA3/St cells. Thus soluble CD44 most likely acts by competitively disrupting an interaction involving hyaluronan.

A particularly striking finding of this study was the failure of TA3/St transfectants overexpressing soluble CD44 to form ascites tumors. For each of the three soluble CD44 transfectants tested, growth took place for several days in the peritoneum subsequent to inoculation. However, the rate of growth of the soluble CD44 transfectants was slower than for controls and the former cells went into G_1 arrest; the control cells, however, continued to increase in number to a point that became fatal for the host animals. Growth of the soluble CD44-transfected cells not only ceased but the numbers of cells in the ascites decreased back to an insignificant level. Depending on the particular transfectant, 3 to 14 million cells per mouse were lost from the peritoneum between 5 and 15 days postinoculation, implying that the soluble CD44 transfectants not only went into G_1 arrest but also subsequently died and were cleared from the peritoneal cavity. In a parallel study, we have compared the ability of the soluble CD44-transfected and vector-transfected TA3/St cells studied herein to form metastases in the lung after intravenous injection.[17] In that study, overexpression of soluble CD44 was shown to induce apoptosis subsequent to entry of the cells into lung tissue, and consequently formation of metastatic nodules was dramatically inhibited. In the current study it is also probable that the soluble CD44 transfectants became apoptotic, although we were unable to capture the cells for analysis during the window of time between appearance of apoptotic characteristics and clearance of the cells from the ascites. TA3/St cells transfected with mutated soluble CD44 (R43A) behaved like vector-transfected controls (Figure 3), indicating that an hyaluronan-mediated interaction is involved in these effects of soluble CD44 on growth.

Interestingly, the cell number reached in the peritoneum for the soluble CD44 transfectants, 5 to 7 days after inoculation, was sufficient for widespread attachment to the peritoneal wall to occur in the case of the controls. However, no attachment of soluble CD44 transfectants was detected. This observation suggests that perturbed hyaluronan-CD44 interactions lead both to altered growth characteristics within the ascites and to inhibition of peritoneal wall implantation. Previous studies have also implicated interactions between tumor cell surface CD44 and mesothelial cell-derived hyaluronan in tumor cell attachment to the peritoneal wall.[4,25,26]

Although decreased attachment of the soluble CD44 transfectants to the peritoneal wall is consistent with past findings, the altered growth characteristics of soluble CD44 transfectants within the ascites were not predicted. Overproduction of soluble CD44 could influence any one of several events necessary for ascites tumor growth. For example, hyaluronan binds to fibrinogen;[27] thus, excess soluble CD44 may disrupt formation in the ascites of a provisional matrix rich in fibrin and hyaluronan that is important for tumor progression.[2,3] Hyaluronan-CD44 interactions may also be involved in angiogenesis,[28,29] in which case soluble CD44 could again be potentially disruptive. Consequently we attempted to determine whether or not perturbation of endogenous tumor cell surface hyaluronan interactions by soluble CD44 gives rise to direct inhibitory effects on tumor cell growth. We have shown that the soluble CD44 transfectants, but not the mutant soluble CD44 transfectant, have lost their ability to exhibit anchorage-independent growth in soft agar, a commonly used indicator of the transformed state of cells.[30,31] Thus it would seem that endogenous hyaluronan produced by the tumor cells themselves serves an important function in anchorage-independent growth. This conclusion is supported by recent experiments showing that increased expression of hyaluronan, driven by transfection with cDNA for hyaluronan synthase, leads to acquisition of the ability to grow in soft agar.[19] However, it is unlikely that the effect of overexpression of soluble CD44 is because of changes in hyaluronan synthesis because none of the parent or transfected cell lines produce large amounts of hyaluronan. Rather, it is more likely that soluble CD44 disrupts the organization of endogenous pericellular hyaluronan with respect to its interactions with CD44 or other hyaluronan-binding proteins that are important for the transformed behavior of the parent and control cells, eg, CD44-mediated docking of MMP-9 (see below). Also, *in vivo*, both parent and soluble CD44 transfectants induce high hyaluronan levels in surrounding stromal tissue,[17] indicating that this is not the underlying difference in their behavior *in vivo*.

Recent work from one of our laboratories[21] has demonstrated binding of MMP-9 to CD44 at the surface of TA3/St murine mammary carcinoma and MC human melanoma cells. This binding of MMP-9 to CD44 is dependent on hyaluronan-induced clustering of CD44 in the plasma membrane. Overexpression of soluble CD44 disrupts clustering of endogenous membrane CD44 and thus inhibits complex formation with MMP-9. Complex formation between CD44 and MMP-9 has also been observed in other mammary carcinoma cell lines.[32] Docking of MMP-9 at the surface of TA3/St cells promotes its activity, possibly via protection from tissue inhibitors of MMPs, which in turn leads to enhanced tumor invasion and angiogenesis.[21,33] Cell surface-bound MMP-9 acts, at least in part, by activating latent transforming growth factor-β1 (TGF-β) which then stimulates new blood ves-

sel formation *in vitro* and *in vivo*.[33] In similar fashion to solid tumors, ascites tumor growth is accompanied by extensive angiogenesis within the peritoneal wall.[3] Thus, it is possible that TGF-β, activated in the above manner by MMP-9, stimulates peritoneal angiogenesis and thus ascites tumor growth. However, promotion of angiogenesis would not explain the involvement of hyaluronan in anchorage-independent growth *in vitro*, as discussed above. The effects of TGF-β on growth characteristics are complex but, in many cases, loss of responsiveness to inhibitory effects of TGF-β is associated with malignancy.[34,35] Although TGF-β is usually thought of as a tumor suppressor, it promotes late stages of carcinoma progression.[36,37] TGF-β also induces anchorage-independent growth in fibroblasts[38] and in immortalized, nontumorigenic epithelial cells.[39] Thus it is conceivable that TGF-β is at least partially responsible for the effects seen herein. Alternatively MMP-9, or another metalloproteinase bound to CD44 in an analogous manner, might cause release of a factor from the tumor cell surface that stimulates transformation and/or tumor growth directly.[40] Thus, hyaluronan- and CD44-dependent presentation of a metalloproteinase at the cell surface could explain the role of hyaluronan in tumor cell growth characteristics and the effects of overexpression of soluble CD44 demonstrated in the present study. Irrespective of the underlying mechanism, our findings lead to the conclusion that hyaluronan interactions at the cell surface are, at least under some circumstances, crucial to tumor cell growth characteristics *in vitro* and *in vivo*.

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